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(54) Promoter for transgenic plants.

(57) A full-length transcript promoter from figwort mosaic virus (FMV) is identified and its DNA sequence given. The promoter functions as a strong and uniform promoter for chimeric genes inserted into plant cells. This strong promoter function is exhibited by a histochemical assay in floral buds and by reproductive scores of transgenic plants including the promoter. The promoter preferably includes a 5′ leader sequence that may be from the FMV itself or from a heterologous source with respect to the promoter. The promoter is used in a plant cassette vector, a chimeric gene and in methods for transforming plant cells to obtain transgenic plants, plant cells or seeds incorporating the FMV promoter.

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PROMOTER FOR TRANSGENIC PLANTS

Background of the Invention

This invention relates in general to plant genetic engineering, and more particularly, to a novel promoter for obtaining constitutive and uniform expression of chimeric genes in plants. This invention also relates to transgenic plants and plant cells containing the promoter.

One of the primary goals of plant genetic engineering is to obtain plants having improved characteristics or traits. The type and number of these characteristics or traits are innumerable, but may include virus resistance, insect resistance, herbicide resistance, enhanced stability or improved nutritional value, to name a few. Recent advances in genetic engineering have enabled researchers in the field to incorporate heterologous genes into plant cells to obtain the desired qualities in the plant of choice. This permits advantageous genes from a source different than the transformed plant to be incorporated into the plant's genome. This new gene can then be expressed in the plant cell to exhibit the new trait or characteristic.

In order for the newly inserted gene to express the protein for which it codes in the plant cell, the proper regulatory signals must be present and in the proper location with respect to the gene. These regulatory signals include a promoter region, a 5′ non-translated leader sequence and a 3′ polyadenylation sequence. The promoter is a DNA sequence that directs the cellular machinery to produce RNA. The promoter region influences the rate at which the RNA product of the gene and resultant protein product of the gene is made. The 3′-polyadenylation signal is a non-translated region that functions in plant cells to cause the addition of polyadenylate nucleotides to the 3′ end of the RNA to stabilize the RNA in the cytoplasm for subsequent translation of the RNA to produce protein.

It has previously been shown that certain promoters are able to direct RNA synthesis at a higher rate than others. These are called strong promoters. Certain promoters have also been shown to direct RNA production at higher levels only in particular types of cells and tissues. Those promoters that direct RNA production in many or all tissues of a plant are called constitutive promoters.

Previous work had shown that the 35S promoter from the cauliflower mosaic virus (CaMV35S) was the strongest constitutive promoter known in plants (Odell et al., 1985; Jensen et al., 1986; Jefferson et al., 1987; Kay et al., 1987; Sanders et al., 1987). This had been shown by demonstrating measurable levels of reporter gene proteins or mRNAs in extracts prepared from the leaves, stems, roots and flowers of transgenic plants. As a result, the CaMV35S promoter has been widely used by scientists in the field of plant genetic engineering.

Although the CaMV35S promoter appeared to be a strong, constitutive promoter in assays involving cell extracts, detailed histological analysis of a reporter gene product that is detectable at the cell and tissue level showed a rather high degree of variability of expression of this gene product. This histological analysis revealed an unknown and unexpected variability in the expression of a gene product driven by the CaMV35S promoter. This variable level and site of expression is believed to have two primary causes. The first is that variability is an intrinsic property of the CaMV35S promoter. The second is that the variability is caused by the position that the CaMV35S promoter driven DNA sequence is integrated into the genome of the transformed plant. When a gene is introduced into a plant cell, the new DNA becomes incorporated at random locations in the plant DNA. This variability in location or insert position leads to a variation in the level of promoter activity and protein production from individual transformants. As a result, a large number of individual transgenic plants must be assayed to find those that produce the highest levels of gene product in most or all of the tissues of the plants. Even the presumed strong, constitutive CaMV35S promoter is subject to the effect of insertion position variability and its use requires that a relatively large number of transformed plants be screened to find ones having appropriate levels of gene expression. Thus, it is clear that a need exists in plant genetic engineering for promoters that express high levels of chimeric gene product, but that is less subject to the wide variation in tissue level expression due to intrinsic properties of the promoter or caused by the effect of insertion position in transgenic plant DNA.

Other caulimoviruses, a group of double-stranded DNA viruses to which the cauliflower mosaic virus belongs, were considered as a potential source for such a promoter. Two caulimoviruses that are distantly related to CaMV have been previously described. The figwort mosaic virus (FMV) was described by Richins et al. (1987) and the carnation etched ring virus (CERV) was described by Hull et al. (1986). The DNA sequence and predicted gene organization of each of these two viruses were similar enough to the CaMV to permit Richins et al. to speculate as to the locations of the FMV and CERV homologues of the CaMV35S promoter. There was, however, little conservation of DNA sequences in these presumptive promoter

regions and no confirming RNA transcript analysis had been carried out to provide a demonstration of the exact location of the promoter sequences, much less a showing that a promoter from FMV would provide an increased and more uniform level of expression of a chimeric gene in plants.

It is therefore a primary object of the present invention to provide a promoter for use in transgenic plants that exhibits an increased and more uniform level of expression of a gene product driven by the promoter than that exhibited by previously known plant promoters.

It is another object of the present invention to provide a promoter for use in transgenic plants that is less affected by insertion position effects than previously known and used plant promoters.

It is a further object of the present invention to provide a promoter for use in transgenic plants that exhibits a higher level of expression of a gene product driven by the promoter in many of the tissues and cells of the plant, particularly the floral buds, than that exhibited by previously known plant promoters.

It is yet another object of the present invention to provide such a promoter for the expression of a chimeric gene in plants that is obtained from the full-length transcript of the figwort mosaic virus.

Other and further objects of the invention will be made clear or become apparent from the following description and claims when read in light of the accompanying drawings.

Brief Description of the Drawings

Figure 1 shows the DNA sequence containing the full-length transcript promoter from the figwort mosaic virus including a 5′ leader sequence and a small amount of 3′ flanking DNA.

Figure 2 shows a physical map of pMON721.

Figure 3 shows a physical map of pMON1573.

Figure 4 shows a physical map of pMON977.

Figure 5 shows a physical map of pMON981.

Figure 6 shows a physical map of pMON994.

Figure 7 shows the steps employed in the preparation of pMON994.

Figure 8 shows a physical map of pMON996.

Figure 9 shows the steps employed in the preparation of pMON996.

Figure 10 shows a restriction map of the T-DNA regions of the *Agrobacterium tumefaciens* strain pTiT37 plasmid which was disarmed to create the ACO *Agrobacterium* strain.

Figure 11(a) and (b) is a color photograph showing the presence of GUS activity in a tobacco flower bud transformed with the β-glucuronidase gene driven by the enhanced CaMVe35S promoter (a) and the FMV full-length transcript promoter (b).

Figure 12 shows the reproductive scores of transgenic plants containing mutant EPSPS under the control of the FMV full-length transcript promoter (PMON996) or CaMVe35S promoter (PMON899) after glyphosate application.

Summary of the Invention

It has been discovered that the full-length transcript promoter from the figwort mosaic virus (FMV) functions as a strong and uniform promoter for chimeric genes inserted into plant cells, particularly in the cells comprising the floral buds. The resulting transgenic plant expresses the protein encoded by the inserted gene at a higher and more uniform level throughout the tissues and cells of the transformed plant than the same gene driven by an enhanced CaMV35S promoter. The DNA sequence of the promoter is located between nucleotides 6368 and 6930 of the FMV genome. A 5′ non-translated leader sequence is preferably coupled with the promoter. The leader sequence can be from the FMV genome itself or can be from a source other than FMV.

Other aspects of the invention include use of the FMV promoter in a method for transforming plant cells, a cassette vector including the FMV promoter, a chimeric gene including the FMV promoter sequence and transgenic plants, plant cells and seeds incorporating the FMV promoter in a chimeric gene.

Detailed Description of the Preferred Embodiment

The figwort mosaic virus (FMV) is a member of the caulimoviruses which are a group of double-stranded DNA viruses. Other members of this group include the cauliflower mosaic virus (CaMV) and the carnation etched ring virus (CERV). The CaMV and its promoter sequences are well-known in the literature (Gardner et al. 1981; Hohn et al. 1982; Guilley et al. 1982). The entire nucleotide sequence of the FMV DNA has been elucidated and reported by Richins et al. (1987). Richins et al. reported two intergenic regions in the FMV genome; a large intergenic region located between open reading frames (ORF) VI and VII and a small intergenic region located between ORFs V and VI. Richins et al. proposed that a promoter sequence analogous to the CaMV35S promoter, the major mRNA transcript promoter of the CaMV, was located in the large intergenic region of the FMV genome, but no confirming RNA transcript analysis had been carried out to provide a demonstration of the exact location of the transcriptional start and, consequently, the promoter sequence.

One aspect of the present invention includes isolation of the promoter for the full-length transcript from the figwort mosaic virus and the determination of the sequence of this promoter. The promoter preferably includes a 5′ leader sequence that may be from the FMV promoter sequence itself or from a source heterologous with respect to the promoter.

The novel promoter of the instant invention was isolated from a small DNA fragment from a complete, full-length clone of FMV DNA. A plasmid, pFMVSc3, was obtained from Dr. R.J. Shepherd of the University of Kentucky. The nucleotide sequence of the FMV DNA and the organization of the FMV genome are given in Richins et al. (1987). This plasmid contains the complete DNA from FMV as adapted for growth on solanaceous hosts as described in Shepherd et al. (1987). As a result of the adaptation of the FMV DNA for growth on solanaceous hosts, the FMV DNA is believed to have undergone a number of mutations at the nucleotide level. In the description and examples that follow FMV DNA from such an adapted strain is used. It is to be understood that the teachings and examples of this invention would also apply to a promoter region isolated from a "wild-type" or non-adapted FMV DNA with similar advantages and results. The original virus was isolated from *Scrophularia californica*. The FMV DNA was cloned into the unique SacI site of pUC13 (Vieira, J. and Messing, J., 1982) to obtain pFMVSc3. The nucleotide sequences shown in the drawing figures accompanying this disclosure that relate to FMV follow the numbering system used by Richins et al.

The FMV promoter sequence was isolated by digesting pFMVSc3 with sspI which cleaves the FMV DNA at several sites including between nucleotides 6367 and 6368 and between nucleotides 6948 and 6949. This releases a 581 base pair (bp) nucleotide fragment that contains a promoter sequence and 18 nucleotides of 5′ non-translated leader sequence corresponding to the full-length transcript promoter of FMV. The nucleotide sequence of this fragment and a small amount of flanking DNA is shown in Fig. 1.

This fragment was purified using the NA-45 membrane method after electrophoretic separation on a 0.8% agarose gel and inserted into plasmid pMON721 that had been cleaved with StuI. A physical map of pMON721 is shown in Fig. 2.

As shown in Fig. 2, plasmid pMON721 contains a StuI site in a multilinker flanked by a HindIII site on one side and a BglII site on the other side. Once the SspI fragment was inserted into pMON721 at the StuI site, the resulting transformed pMON721 plasmids were screened for identification of transformants carrying the presumed FMV full-length RNA transcript promoter fragment oriented in the proper manner. A plasmid identified as pMON1573 was identified as containing the FMV promoter fragment properly oriented so that the presumed 5′ or upstream sequences of the promoter were adjacent to the HindIII site and the untranslated leader sequences terminated at the BglII site. Fig. 3 is a physical map of pMON1573.

Once a plasmid containing the FMV major RNA (full-length) transcript promoter sequence in the correct orientation was isolated, a cassette vector containing this promoter was prepared. A cassette vector is a cloning vector that typically includes all of the necessary elements needed for transformation of plants or plant cells. Typical plant cloning vectors comprise selectable and scoreable marker genes, T-DNA borders, cloning sites, appropriate bacterial genes to facilitate identification of transconjugates, broad host-range replication and mobilization functions and other elements as desired. A cassette vector containing the FMV major RNA transcript promoter of the present invention in a suitable plant transformation vector was prepared by starting with the pMON977 plasmid. A physical map of pMON977 is as illustrated in Fig. 4.

As shown in Fig. 4, pMON977 has the following elements; a 0.93 kb fragment isolated from transposon Tn7 encoding a bacterial spectinomycin/streptomycin resistance gene (Spc/Str) that functions as a marker for selection of the plasmid in *E. coli* and *Agrobacterium* (Fling, M.E., et al. 1985); a 1.61 kb segment of DNA encoding a chimeric kanamycin resistance gene (P-35S/Kan/NOS3′) that permits selection of transformed plant cells (Beck, E., et al. 1982); a 0.75 kb oriV DNA sequence containing the origin of replication from the Rk2 plasmid (Stalker, D.M., et al. 1979) a 3.1 kb segment of pBR322 (ori-322) that provides the origin of replication for maintenance in *E. coli* and the bom site for the conjugational transformation to the *Agrobacterium* cells (Sutliffe, J., 1979); a 0.36 kb segment from pTiT37 (the PvuI to BclI fragment) that carries the nopaline-type T-DNA right border (Fraley et al. 1985); and a 1.15 kb expression cassette consisting of the 0.66 kb enhanced 35S promoter P-e35S (Kay et al. 1987), several unique restriction sites and the 0.7 kb 3′ non-translated region of the pea ribulose bisphosphate carboxylase small subunit E9 gene (E9 3′) (Coruzzi, G., et al., 1984 and Morelli, G. et al., 1985). Plasmid pMON977 was cut with HindIII and BglII to remove the CaMV P-e35S enhanced 35S promoter. A 605bp fragment containing the FMV full-length transcript promoter was excised from pMON1573 with HindIII and BglII and cloned into pMON977 to create pMON981. Plasmid pMON981 thus contains the FMV full-length transcript promoter and the E9-3′ gene (FMV-E9 3′) as an expression cassette. Also included in pMON981

between the FMV promoter and the E9-3′ gene are restriction endonuclous sites for XbaI, BglII and SmaI. A physical map of pMON981 is shown in Fig. 5.

In order to determine that the isolated FMV sequence included the desired promoter region and to demonstrate the effectiveness and utility of the isolated FMV promoter, reporter genes were inserted into plant cassette vector pMON981. The reporter genes chosen were the *E. coli* β-glucuronidase (GUS) coding sequence and the *Arabidopsis* EPSP synthase gene containing a single glycine to alanine substitution which causes this enzyme to be tolerant of glyphosate herbicides.

The *E. coli* β-glucuronidase coding sequence was inserted into the unique BglII site in the FMV-E9 3′ cassette of plasmid pMON981. The GUS gene was excised from pMON637 on an 1885bp BglII to BamHI fragment. The resulting plasmid was denoted pMON994 and contains the GUS gene under control of the FMV promoter. Plasmid pMON994 is shown in Fig. 6 and a flow chart illustrating the development of pMON994 is shown in Fig. 7.

EPSP synthase (5-enolpyruvyl-3-phosphoshikimate synthase; EC:25.1.19) is an enzyme involved in the shikimic acid pathway of plants. The shikimic acid pathway provides a precursor for the synthesis of aromatic amino acids essential to the plant. Specifically, EPSP synthase catalyzes the conversion of phosphoenol pyruvate and 3-phosphoshikimic acid to 5-enolpyruvyl-3-phosphoshikimic acid. A herbicide containing N-phosphonomethylglycine inhibits the EPSP synthase enzyme and thereby inhibits the shikimic acid pathway of the plant. The term "glyphosate" is usually used to refer to the N-phosphonomethylglycine herbicide in its acidic or anionic forms. Novel EPSP synthase enzymes have been discovered that exhibit an increased tolerance to glyphosate containing herbicides. In particular, an EPSP synthase enzyme having a single glycine to alanine substitution in the highly conserved region having the sequence: -L-G-N-A-G -T-A- located between positions 80 and 120 in the mature wild-type EPSP synthase amino acid sequence has been shown to exhibit an increased tolerance to glyphosate and is described in the commonly assigned pending patent application entitled "Glyphosate-Tolerant 5-Enolpyruvyl-3-Phosphoshikimate Synthase" having U.S. serial number 931,492, the teachings of which are hereby incorporated by reference hereto. Methods for transforming plants to exhibit glyphosate tolerance are discussed in the commonly assigned U.S. patent application entitled "Glyphosate-Resistant Plants," Serial No. 879,814 filed July 7, 1986, the disclosure of which is specifically incorporated herein by reference. A glyphosate-tolerant EPSP synthase plant gene encodes a polypeptide which contains a chloroplast transit peptide (CTP) which enables the EPSP synthase polypeptide (or an active portion thereto) to be transported into a chloroplast inside the plant cell. The EPSP synthase gene is transcribed into mRNA in the nucleus and the mRNA is translated into a precursor polypeptide (CTP/mature EPSP synthase) in the cytoplasm. The precursor polypeptide is transported into the chloroplast.

The EPSP synthase gene containing a single glycine to alanine mutation obtained from mutated *Arabidopsis thaliana* gene sequence was also inserted into the FMV-E9 3′ cassette vector of plasmid pMON981. Plasmid pMON981 was cut with XbaI and SmaI. The *Arabidopsis* EPSP synthase gene is located on plasmid pMON897. Plasmid pMON897 is obtained by excising the *Arabidopsis* EPSP synthase gene (AEPSPS) in pMON600 by cutting with ClaI and EcoRI. This fragment is inserted into pMON855 which includes a multilinker containing sites for EcoRI, ClaI and XbaI. Plasmid pMON855 is cut with ClaI and EcoRI and the *Arabidopsis* EPSP synthase fragment isolated from pMON600 is inserted. The resulting plasmid is pMON897. Plasmid pMON897 was then cut with EcoRI and the ends were filled in using Klenow polymerase and then cut with XbaI and the *Arabidopsis* EPSP synthase gene was excised as a 3881 bp fragment. The *Arabidopsis* EPSP synthase gene was then cloned into pMON981 digested with XbaI and SmaI to create pMON996. A physical map of pMON996 is shown in Fig. 8 and a flow chart illustrating the development of pMON996 is shown in Fig. 9.

Once the FMV-E9 3′ cassette vector containing the desired reporter gene is prepared, the vector can then be inserted into suitable *Agrobacterium* strains for *Agrobacterium* mediated transformation into plants or plant cells. The *Agrobacterium tumefaciens* strain to be used preferably contains a disarmed Ti plasmid. Two particularly useful strains are *Agrobacterium tumefaciens* strain A208 carrying the disarmed Ti plasmid pTiC58 derivative, pMP90RK (Koncz and Schell, 1986) and the ACO *Agrobacterium tumefaciens* strain carrying the disarmed pTiT37-CO nopaline type plasmid.

The *A. tumefaciens* strain 208 carrying the disarmed pMP90RK plasmid does not carry the T-DNA phytohormone genes and therefore cannot cause crown gall disease. When this strain is used for plant transformations, the vector plasmid is introduced into the *Agrobacterium* by the triparental conjugation system (Ditta et al. 1980) using the helper plasmid pRK2013. The vectors are transferred to plant cells by the vir functions encoded

by the disarmed pMP90RK Ti plasmid. Analysis of transformants suggest that the vector is opened at the pTiT37 right border sequence and the entire vector sequence is inserted into the host plant chromosome. The pMP90RK Ti plasmid is probably not transferred to the plant cell but remains in the *Agrobacterium*.

Figure 10 shows a restriction map of the T-DNA regions of the *Agrobacterium tumefaciens* strain pTiT37 plasmid which was disarmed to create the ACO *Agrobacterium* strain. This strain carries the disarmed pTiT37-C0 nopaline type plasmid. The hatched boxes in Fig. 10 show the segments of the Ti plasmid DNA which were used to provide homology for recombination and replacement of the T-DNA. The T-DNA segment was replaced by the Tn601 bacteria kanamycin resistance gene (Kn^R) segment joined to the OriV and pBR322 segment homologous to the vectors described above. The recombination between the disarmed pTiT37-CO and plant cassette vector takes place through the pBR322 oriV area of homology resulting in the hybrid T-DNA which contains the entire DNA of the cassette vector plasmid. On cultivation of the *Agrobacterium* with plant cells, the hybrid T-DNA segment between the left and right borders is transferred to the cells and integrated into the genomic DNA.

Once the vector has been introduced into the disarmed *Agrobacterium* strain, the desired plant can then be transformed. Any known method of transformation that will work with the desired plant can be utilized. These methods include the leaf disc method of Horsch et al. (1984) and as adapted by Fry et al. (1986) for *Brassica napus*. Also conceived to be within the scope of the present invention is the use of DNA fragments or vectors including the FMV promoter sequences coupled with heterologous DNA sequences in the transformation of plants utilizing techniques such as electroporation or particle gun transformation.

Suitable plants for the practice of the present invention include, but are not limited to, soybean, cotton, alfalfa, oilseed rape, flax, tomato, sugar beet, sunflower, potato, tobacco, maize, wheat, rice and lettuce.

The effectiveness of the FMV promoter was determined by comparison studies with the enhanced CaMV35S promoter. In one study, pMON994 containing the FMV promoter including the 5′ non-translated leader sequence from FMV fused to the β-glucuronidase reporter gene and the E9-3′ non-translated polyadenylation region from pea was introduced into tobacco using the leaf disc method of Horsch et al. (1984) and transgenic plants obtained.

Tobacco (*Nicotiani tabacum var. samsun*) leaf disks with diameters of about 6mm (¼inch) were taken from surface sterilized tobacco leaves. These were cultivated on MS104 agar medium for two days to promote partial cell wall formation at the wound surfaces. They were then submerged in a culture of *A. tumefaciens* cells containing both pMON994 and pMP90RK which had been grown overnight in Luria broth at 28° C, and shaken gently. The cells were removed from the bacterial suspension, blotted dry, and incubated upside down on filter paper placed over "nurse" cultures of tobacco cells as described by Horsch (1980). After two or three days, the disks were transferred to petri dishes containing MS media with 500μg/ml carbenicillin with no nurse culture.

Control tissue was created using *A. tumefaciens* cells containing the helper plasmid pMP90RK and a different plant transformation vector, pMON505, which contained a T-DNA region with a NOS/NPTII/NOS kanamycin resistance gene and a NOS selectable marker gene identical to pMON994, but without the FMV/β-glucuronidase gene.

Within ten days after transfer to the MS media, actively growing callus tissue appeared on the periphery of all disks on both the control and transformed plates.

Transformed tobacco plants were produced by regeneration from the above-described transformed leaf disks by the procedure described by Horsch, et al. (1985). The transformed plants obtained contained the pMON994 vector which contains the FMV promoter fused to the β-glucuronidase gene.

The same procedure as described above was utilized to obtain transformed tobacco plants containing the enhanced CaMV35S (CaMVe35S or P-e35S) promoter fused to the β-glucuronidase reporter gene and the E9-3′ non-translated polyadenylation region from pea.

A second study involved obtaining transformed canola plants (Brassica napus) carrying the *Arabidopsis* EPSP synthase gene containing a single glycine to alanine substitution at amino acid 101 driven by either the FMV promoter or the CaMVe35S promoter. The pMON996 plasmid carrying the *Arabidopsis* EPSP synthase gene directed by the FMV promoter was introduced into canola by the method of Fry et al. (1986). Four terminal internodes from plants just prior to bolting or in the process of bolting, but before flowering were removed and surface sterilized in 70° v/v ethanol for one minute, 2% w/v sodium hypochlorite for twenty minutes, and rinsed three times in sterile distilled water. Stem segments were cut into 5mm discs (Stringam 1977) and placed in a sterile 15x100mm petri plate, noting the orientation of the basal end. The discs were inoculated for five minutes by pouring two to four milliliters of an overnight culture of the ACO *A. tumefaciens* strain

containing pMON996 as previously described over the discs in the petri plate and then blotted dry by placing sterile filter paper in the petri plate and turning the plate over to absorb any excess bacteria. The stem discs were placed basal side down on feeder plates on medium containing 1/10x standard MS salts, B5 vitamins, 3% sucrose, 0.8% agar, pH 5.7, 1mg/l BA and 1.4ml TXD feeder cells (Horsch et al. 1985).

After a two to three day coculture period, stem discs were transferred, five to a deep dish petri plate (25 x 100mm) containing the same medium with standard MS salts, 1mg/l BA, 500 mg/l carbenicillin, 0.3 mm arginine, and 100 mg/l kanamycin for selection. At three weeks the stem explants were transferred to fresh plates containing the same medium. Culture of the explants was in a growth room under continuous cool white light at 26°C. Shoots that developed in the next one to three week period were excised from the stem explants, dipped in Rootone® and placed in 2½ inch pots containing water saturated Metro Mix 350 in closed GAF containers for ten days in a chamber with a constant temperature of 21°C and a 16 hour photoperiod. The shoots are assayed for the presence of kanamycin resistance immediately after being excised from the stem explant while still sterile.

This same procedure was used to obtain transformed canola plants containing the enhanced CaMVe35S promoter fused to the *Arabidopsis* EPSP synthase gene by inoculating the stem segment discs with ACO *Agrobacterium tumefaciens* strain containing pMON899.

Example 1

Transformed plants containing the GUS gene driven by either the FMV full-length promoter or the enhanced CaMVe35S promoter were assayed using a histological staining procedure to determine GUS activity in the transformed cells. The results of these assays on plants transformed with pMON994 (FMV/GUS/E9) were compared to the results of the same assays performed on plants transformed with pMON977 (CaMVe35S/GUS/E9).

The histochemical assay of the tobacco plants containing the FMV/GUS/E9 and CaMVe35S/GUS/E9 constructs involved examination of young flower bud (10mm) sections of the transformed plants to determine GUS activity. The flower bud section of the transformed plant was prepared by using a razor blade to free-hand section the plant tissue into sections less than 0.5mm in thickness. The tissue was then placed in excess X-gluc solution so that the section was fully covered. Pulling a vacuum on the sections may aid in penetration of the X-gluc solution. A 50ml X-gluc solution was prepared by combining 25ml of 0.2M $NaPO_4$ buffer pH 7.0, 24.0ml dH_2O, 0.25ml 0.1M $K_3[Fe(CN)_6]$, 0.25ml 0.1M $K_4[Fe(CN)_6]$ and 0.5ml 1M EDTA,pH7.0. To this solution, 50mg of X-gluc (5-bromo-4-chloro-3-idolyl-β-glucuronide) obtained from Research Organics (Cleveland, Ohio) was added and stirred until dissolved. The solution was then preferably sterilized by filtration. The flower bud sections in the X-gluc solution were then placed at 37°C for 2-4 hours. Care was taken to prevent evaporation of the solution. After the incubation period, the sections were rinsed with phosphate buffer, or distilled H_2O, and the sections were examined immediately with a dissecting scope or compound microscope. If there is interference from the pigments, the tissue can be fixed in FAA solution (85ml 50% ethanol, 5ml glacial acetic acid and 10ml formalin) for 24 hours. Problems with phenolics can be mitigated by the addition of sodium metabisulfite to 20mM to the staining solution just prior to staining. Figure 11 illustrates the results of the histological staining assay of the FMV containing GUS construct and the CaMVe35S containing GUS construct, respectively.

A positive test for the presence of GUS activity is shown by a blue coloration appearing in the tissue of the assayed plant section. In Fig. 11, a color photograph of the stained section of a tobacco flower bud transformed with the β-glucuronidase gene driven by the enhanced CaMVe35S promoter (a) and the FMV full-length promoter (b) is shown. Fig. 11(a) exhibits a typical staining profile for a CaMVe35S promoter driven GUS gene with staining in some tissues and no staining in other tissues within a single transgenic plant. The level of expression in those tissues expressing the GUS gene is considered fair. In Fig. 11(b), tissue from a plant transformed with the FMV promoter driven GUS gene shows that the transformed plant is showing much higher levels of GUS expression and a more uniform pattern of expression throughout the tissue and cells. This is illustrated by the predominant blue coloration throughout the section.

The distribution of expression and the number of highly expressing transgenic plants obtained show that the FMV promoter is superior in tissue distribution and uniformity of expression when compared to the best enhanced CaMV promoter containing transformed plants. Greater than 90% of the FMV/GUS containing transformed plants showed very strong GUS expression and that the staining was uniform from plant to plant and tissue to tissue. This staining is consistently as good in the FMV containing plants as that in the best enhanced CaMV/GUS plants identified.

Example 2

Transgenic plants containing the *Arabidopsis* EPSP synthase gene containing a single glycine to alamine mutation at nucleotide 101 driven by either the FMV promoter or the CaMVe35S promoter were obtained and analyzed for resistance to glyphosate. The transgenic plants containing the *Arabidopsis* EPSP synthase gene (as described) directed by the FMV promoter contained pMON996 while those plants containing the enhanced CaMVe35S promoter contained pMON899. These transgenic plants were planted and the seed from the R_0 plants harvested, threshed and dried before planting for a glyphosate spray test. The progeny were planted in 4-inch square pots of Metro 350 and three types of slow release fertilizers. A goal of twenty seedlings from each R_0 plant is desirable for testing. Germination frequency is usually high but overplanting ensures that twenty seedlings are present. The plants were thinned down by selecting the twenty most vigorous and erect seedlings seven to ten days after planting. A negative control (non-transformed, "Westar" variety) was planted at the same time to maintain quality and display the results. The plants were maintained and grown in a greenhouse environment. A sixteen-hour photoperiod and a temperature of 21° C (day) and 15° C (night) was maintained. Water soluble Peters Pete Lite fertilizer with an analysis of 20-19-18 was applied once per week or as needed.

Two plants from each R_0 progeny were not sprayed and served as controls to compare and measure the glyphosate tolerance. When the remaining plants reached the six to eight leaf stage, usually 20 to 28 days after planting, glyphosate was applied at a rate equivalent to 0.28 Kg/ha. Low rate technology using low volumes has been adopted. A volume of ten imperial gallons for 0.28 Kg/ha of glyphosate is standard in field tests. A laboratory test sprayer had been calibrated to deliver a consistent rate equivalent to field conditions.

Results of reproductive evaluations are shown in Fig. 12. These calculations are based upon a numerical scoring system relative to nonsprayed controls. Reproductive scores are examined at 28 days after spraying and are based upon six distinct conditions in which the main meristem or flowers reacted to the glyphosate. The scale used is:

0 = no floral bud development
2 = floral buds, but aborted prior to opening
4 = flowers without antlers, antlers should protrude past petals
6 = flowers with normal appearing antlers, but sterile
8 = flowers with partially sterile antlers
10 = fully fertile flowers

Figure 12 compares the reproductive scores of the total number of transgenic canola lines containing the FMV promoter with transgenic lines containing the CaMVe35S promoter. As can be seen in Fig. 12, the reproductive scores of three of the seven transgenic lines containing the FMV promoter (pMON996) are better than any of the scores from lines containing the CaMVe35S promoter (pMON899). In fact, the transgenic lines containing pMON899 used in Fig. 12 exhibit the highest levels of glyphosate tolerance among 150 lines previously tested. This demonstrates that the FMV promoter more uniformly expresses a gene product throughout the tissues and cells of the plant, and particularly in the floral buds. It is to be understood that an increased level of expression in the floral buds is important for maximal glyphosate resistance.

The embodiments and examples described above are provided to better elucidate the practice of the present invention. It should be understood that these embodiments and examples are provided for illustrative purposes only, and are not intended to limit the scope of the invention.

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Claims

1. A full-length transcript promoter from figwort mosaic virus.
2. The promoter of claim 1 wherein said promoter has the nucleotide sequence as shown in nucleotides 6368 to 6930 of Figure 1.
3. A promoter of claim 1 further comprising a 5′ non-translated leader sequence from figwort mosaic virus.
4. A promoter of claim 1 further comprising a 5′ non-translated leader sequence from a source heterologous with respect to the promoter.
5. The promoter of claim 3 wherein said promoter has the nucleotide sequence as shown in Figure 1.
6. A method for transforming a plant cell to express a chimeric gene, the improvement comprising a chimeric gene containing a full-length transcript promoter from figwort mosaic virus.
7. A method of claim 6 wherein said promoter includes a 5′ non-translated leader sequence.
8. A method of claim 7 wherein said 5′ non-translated leader sequence is from figwort mosaic virus.
9. A method of claim 7 wherein said 5′ non-translated leader sequence is from a source heterologous with respect to the promoter.
10. A chimeric gene that functions in plant cells comprising:
a full-length transcript promoter from figwort mosaic virus;
a structural DNA sequence that is heterologous with respect to the promoter; and
a 3′ non-translated region which encodes a polyadenylation signal which functions in plant cells to cause the addition of polyadenylate nucleotides to the 3′ end of the RNA.
11. The chimeric gene of claim 10 wherein said promoter further comprises a 5′ non-translated leader sequence.
12. The chimeric gene of claim 11 wherein said 5′ non-translated leader sequence is from figwort mosaic virus.
13. The chimeric gene of claim 11 wherein said 5′ non-translated leader sequence is from a source heterologous with respect to the promoter.
14. A chimeric gene of claim 10 wherein said structural DNA sequence comprises a coding sequence which causes the production of RNA, encoding a chloroplast transit peptide/5-enolpyruvylshikimate-3-phosphate synthase fusion polypeptide, which chloroplast transit peptide permits the fusion polypeptide to be imported into a chloroplast of a plant cell.
15. A transformed plant cell that contains a chimeric gene comprising:
a full-length transcript promoter from figwort mosaic virus;
a structural DNA sequence that is heterologous with respect to said promoter; and
a 3′ non-translated region which encodes a polyadenylation signal which functions in plant cells to cause the addition of polyadenylate nucleotides to the 3′ end of the RNA.
16. A plant cell of claim 15 wherein said promoter further comprises a 5′ non-translated leader sequence.
17. A transformed plant cell of claim 16 wherein said 5′ non-translated leader sequence is from figwort mosaic virus.
18. A transformed plant cell of claim 16 wherein said 5′ non-translated leader sequence is from a source heterologous with respect to the promoter.
19. A transformed plant cell of claim 15 wherein said structural DNA sequence further comprises a coding sequence which causes the production of RNA, encoding a chloroplast transit peptide/5-enolpyruvylshikimate-3-phosphate synthase fusion polypeptide, which chloroplast transit peptide permits the fusion polypeptide to be imported into a chloroplast of a plant cell.
20. A transformed plant cell of claim 19 wherein the coding sequence encodes a glyphosate tolerant 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS).
21. A transformed plant cell of claim 19 wherein the chloroplast transit peptide is from a plant EPSPS gene.
22. A chimeric gene of claim 14 wherein the coding sequence encodes a glyphosate-tolerant 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS).
23. A chimeric gene of claim 14 wherein the chloroplast transit peptide is from a plant EPSPS gene.
24. A plant transformation vector which comprises

a disarmed plant tumor inducing plasmid of Agrobacterium tumefaciens which is capable of inserting a chimeric gene into susceptible plant cells, wherein said chimeric gene comprises a full-length transcript promoter from figwort mosaic virus and a structural DNA sequence that is heterologous with respect to the promoter.

25. A plant transformation vector of claim 24 wherein said promoter further comprises a 5′ non-translated leader sequence.

26. A plant transformation vector of claim 25 wherein said 5′ non-translated leader sequence is from figwort mosaic virus.

27. A plant transformation vector of claim 25 wherein said 5′ non-translated leader sequence is from a source heterologous with respect to the promoter.

28. A transgenic plant which comprises plant cells of any of Claims 15 to 21.

29. A seed from a plant of Claim 28.

FIG. 1


Sspl
TCATCAAAATATTTAGCAGCATTCCAGATTGGGTTCAATCAACAAGGTACGAGCCATATC
6358
6417
AGTAGTTTTATAAATCGTCGTAAGGTCTAACCCAAGTTAGTTGTTCCATGCTCGGTATAG
ACTTTATTCAAATTGGTATCGCCAAAACCAAGAAGGAACTCCCATCCTCAAAGGTTTGTA
6418
6477
TGAAATAAGTTTAACCATAGCGGTTTTGGTTCTTCCTTGAGGGTAGGAGTTTCCAAACAT
AGGAAGAATTCTCAGTCCAAAGCCTCAACAAGGTCAGGGTACAGAGTCTCCAAACCATTA
6478
6537
TCCTTCTTAAGAGTCAGGTTTCGGAGTTGTTCCAGTCCCATGTCTCAGAGGTTTGGTAAT
GCCAAAAGCTACAGGAGATCAATGAAGAATCTTCAATCAAAGTAAACTACTGTTCCAGCA
6538
6597
CGGTTTTCGATGTCCTCTAGTTACTTCTTAGAAGTTAGTTTCATTTGATGACAAGGTCGT
CATGCATCATGGTCAGTAAGTTTCAGAAAAAGACATCCACCGAAGACTTAAAGTTAGTGG
6598
6657
GTACGTAGTACCAGTCATTCAAAGTCTTTTTCTGTAGGTGGCTTCTGAATTTCAATCACC
GCATCTTTGAAAGTAATCTTGTCAACATCGAGCAGCTGGCTTGTGGGGACCAGACAAAAA
6658
6717
CGTAGAAACTTTCATTAGAACAGTTGTAGCTCGTCGACCGAACACCCCTGGTCTGTTTTT
AGGAATGGTGCAGAATTGTTAGGCGCACCTACCAAAAGCATCTTTGCCTTTATTGCAAAG
6718
6777
TCCTTACCACGTCTTAACAATCCGCGTGGATGGTTTTCGTAGAAACGGAAATAACGTTTC
ATAAAGCAGATTCCTCTAGTACAAGTGGGGAACAAAATAACGTGGAAAAGAGCTGTCCTG
6778
6837
TATTTCGTCTAAGGAGATCATGTTCACCCCTTGTTTTATTGCACCTTTTCTCGACAGGAC
ACAGCCCACTCACTAATGCGTATGACGAACGCAGTGACGACCACAAAAGAATTCCCTCTA
6838
6897
TGTCGGGTGAGTGATTACGCATACTGCTTGCGTCACTGCTGGTGTTTTCTTAAGGGAGAT
Sspl
TATAAGAAGGCATTCATTCCCATTTGAAGGATCATCAGATACTGAACCAATATTTCTC
6898
6955
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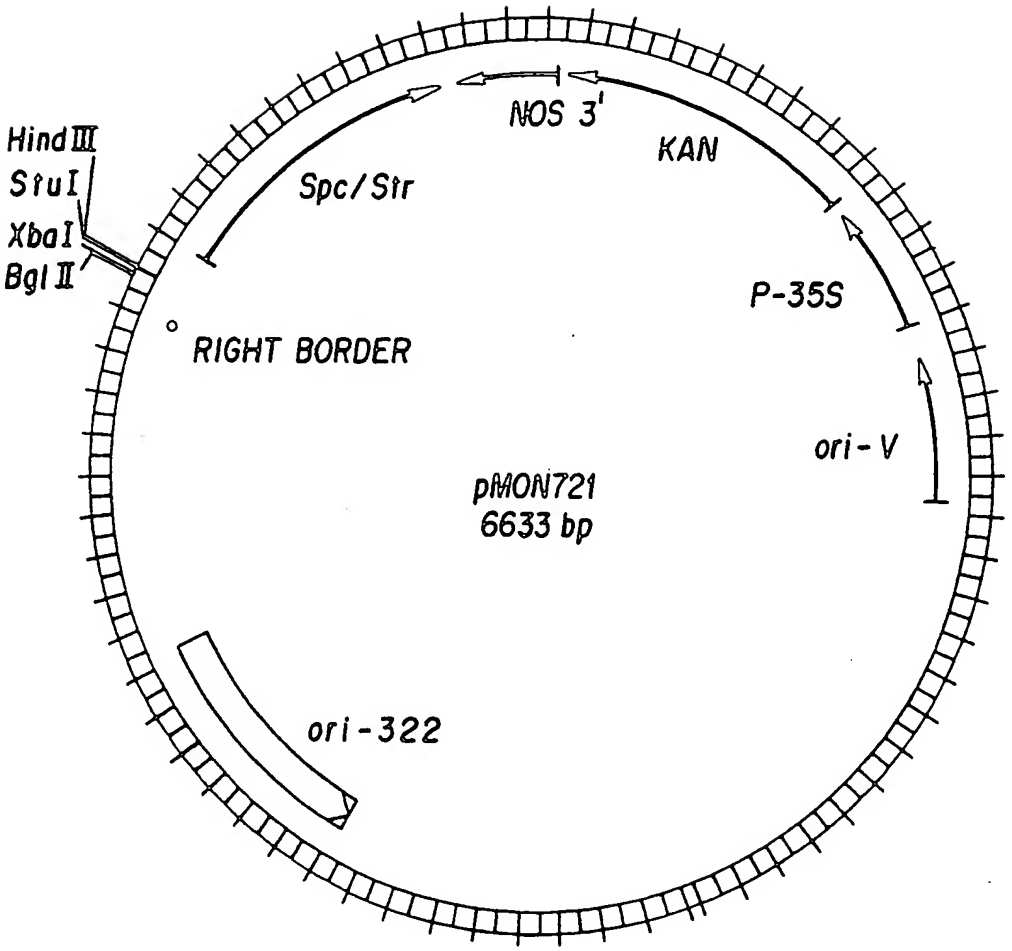
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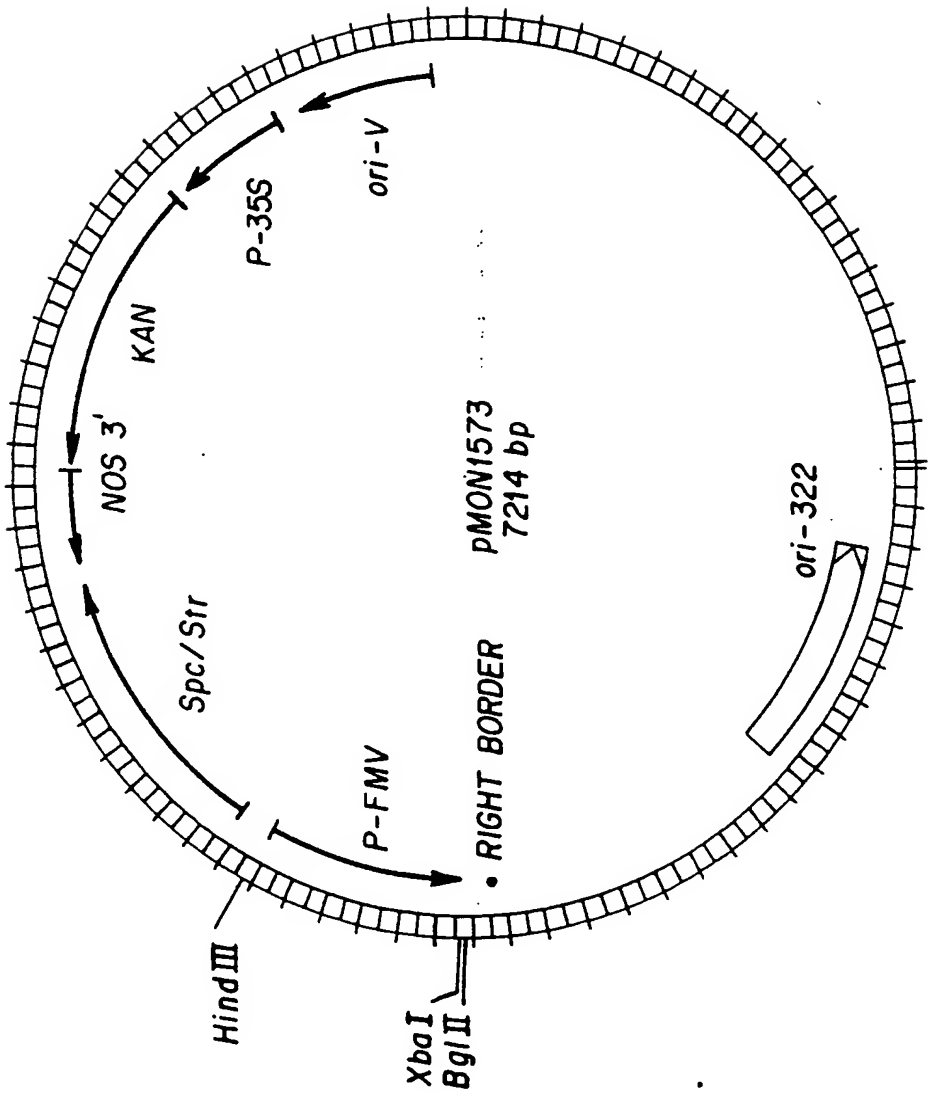
Stu I
Xba I
Bgl II
NOS 3'
KAN
Spc/Str
P-35S
RIGHT BORDER
ori-V
pMON721
6633 bp
ori-322

FIG. 2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P-35S
KAN
NOS 3'
Spc/Str
P-FMV
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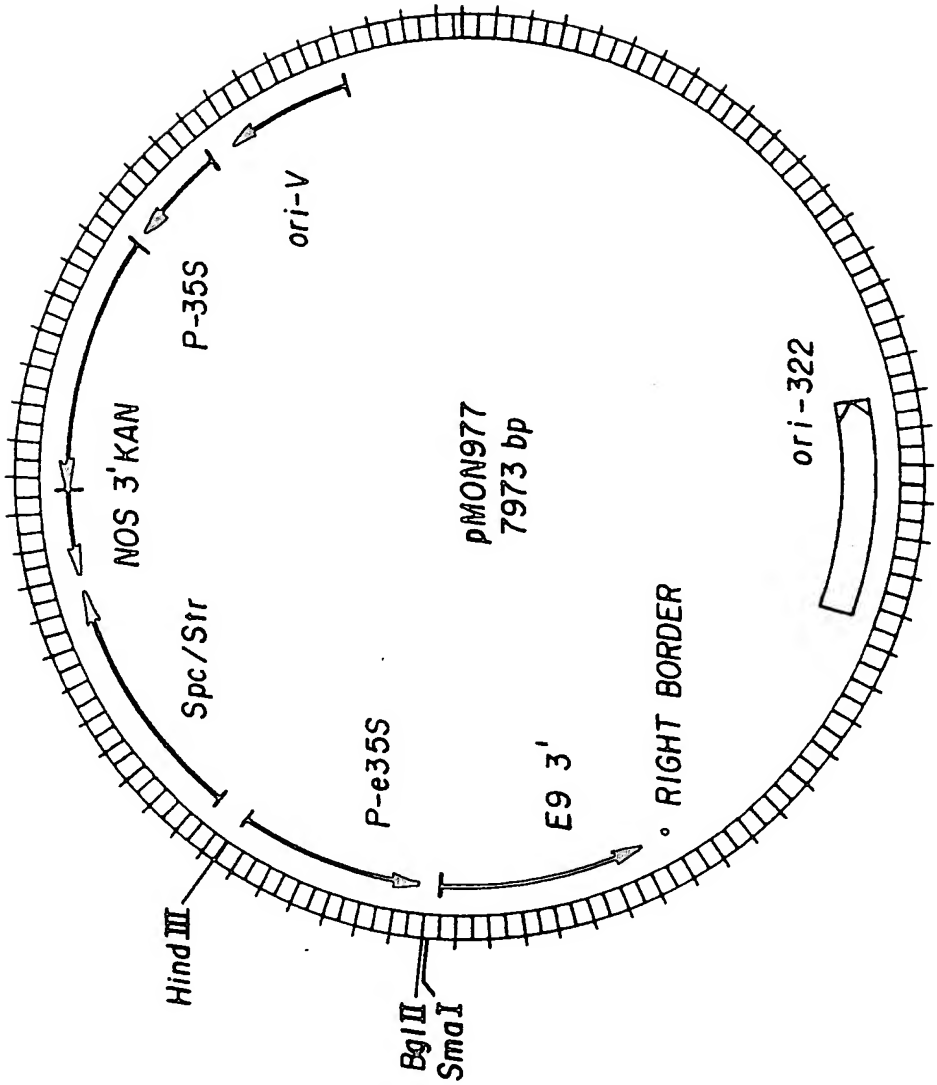
pMON1573
7214 bp
ori-322
HindIII
XbaI
BglII

FIG. 3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P-35S
NOS 3'KAN
Spc/Str
P-e35S
E9 3'
RIGHT BORDER
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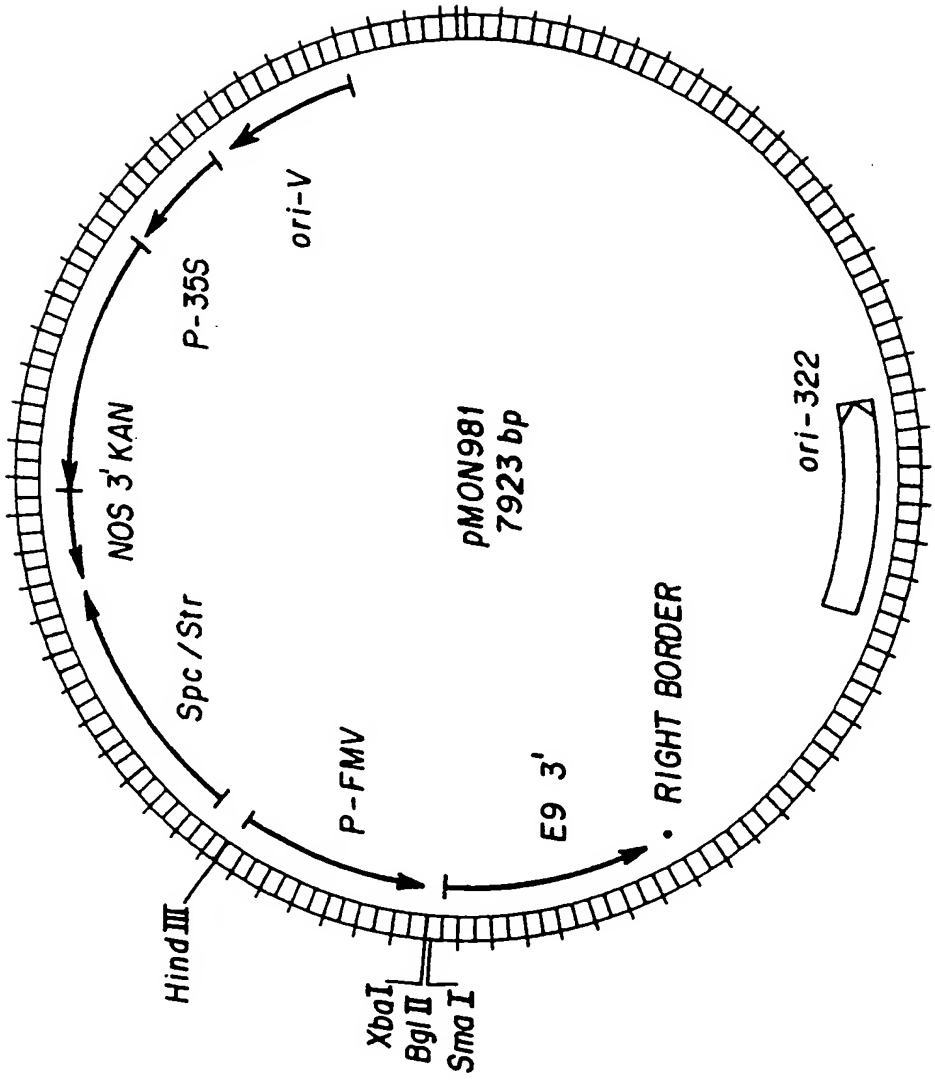
HindIII
BglII
SmaI

FIG. 4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7923 bp
NOS 3' KAN
P-35S
ori-V
Spc/Str
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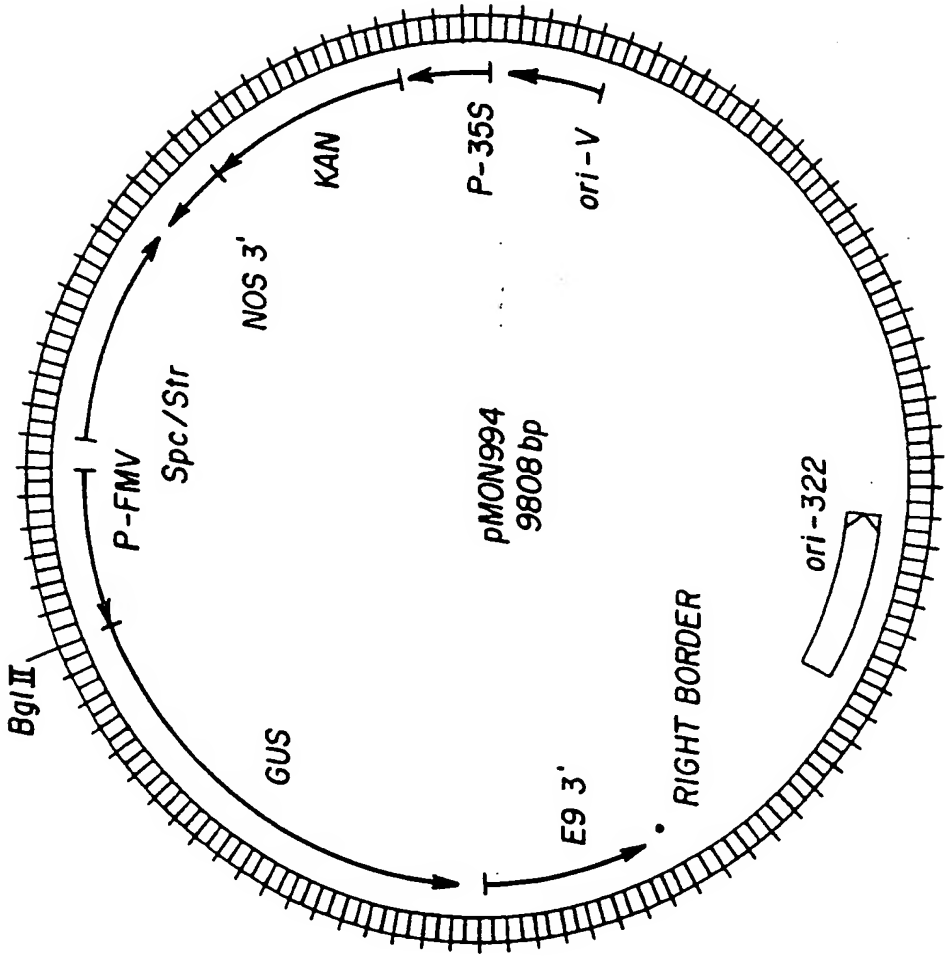
E9 3'
RIGHT BORDER
ori-322
HindIII
XbaI
BglII
SmaI

FIG. 5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P-FMV
Spc/Str
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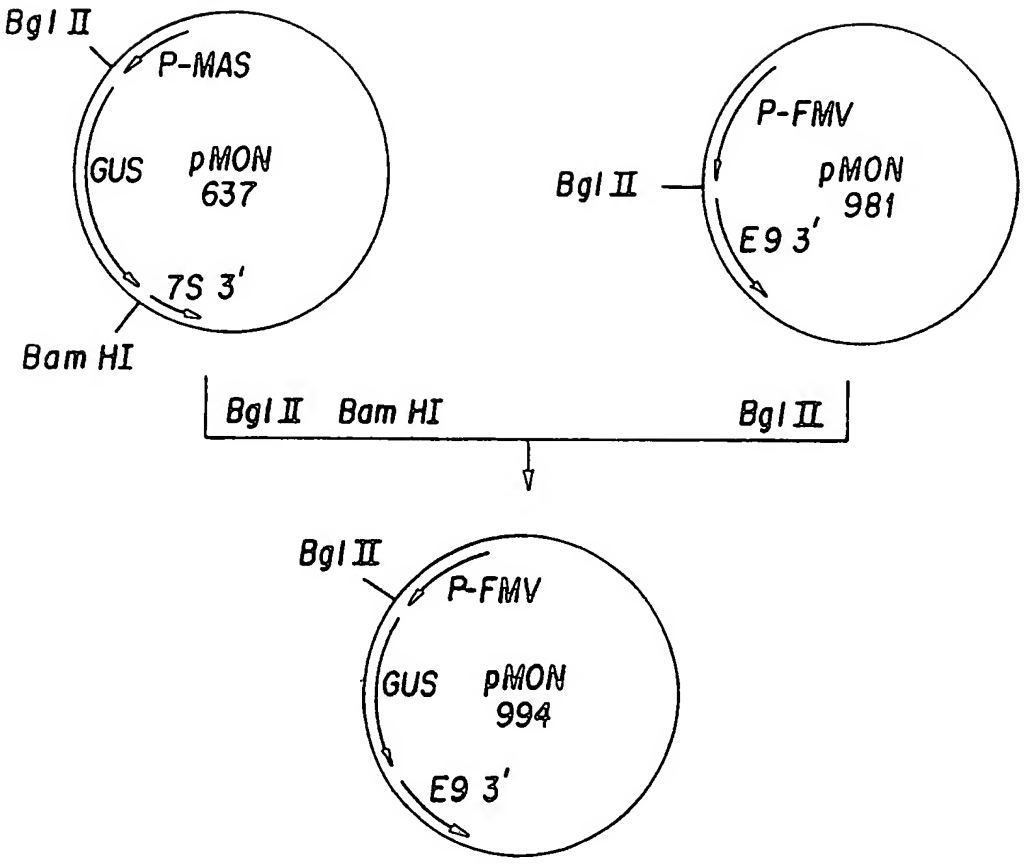
KAN
P-35S
ori-V
pMON994
9808 bp
ori-322
RIGHT BORDER
E9 3'
GUS

FIG. 6


Bgl II
P-MAS
GUS
pMON
637
7S 3'
Bam HI
P-FMV
pMON
981
Bgl II
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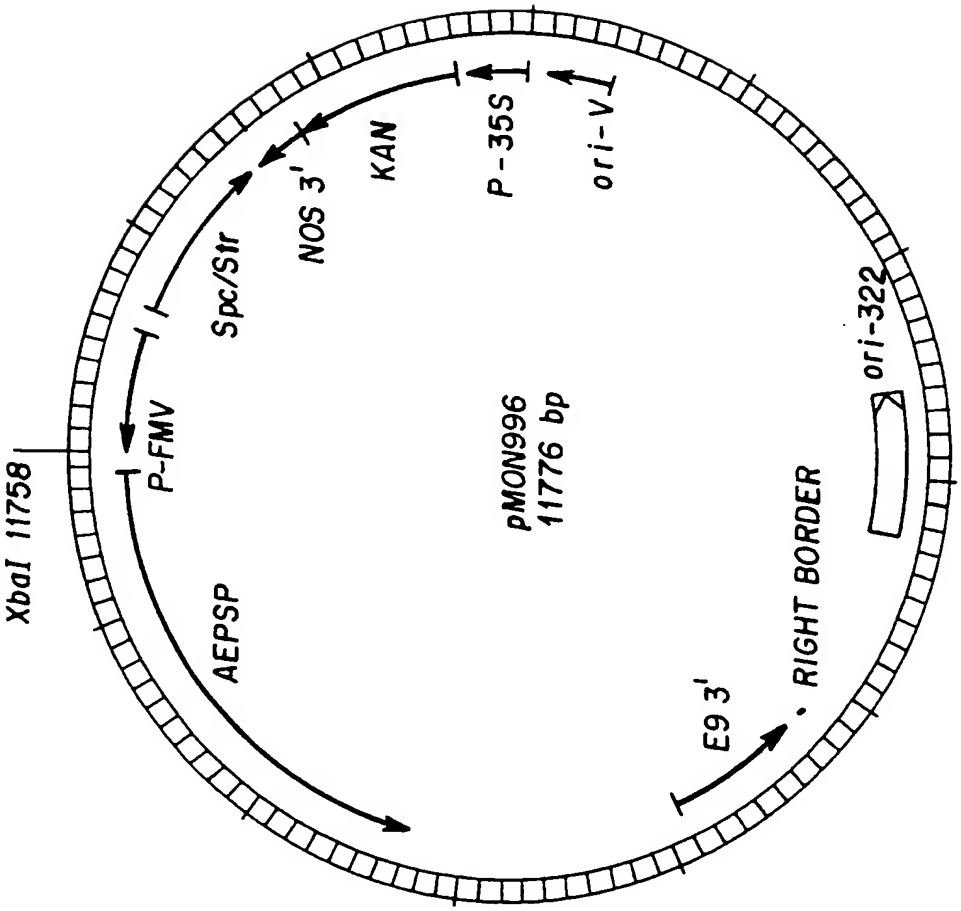
Bgl II Bam HI
Bgl II
Bgl II
P-FMV
GUS
pMON
994
E9 3'

FIG. 7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P-FMV
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Spc/Str
NOS 3'
KAN
P-35S
ori-V
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11776 bp
ori-322
RIGHT BORDER
E9 3'

FIG. 8

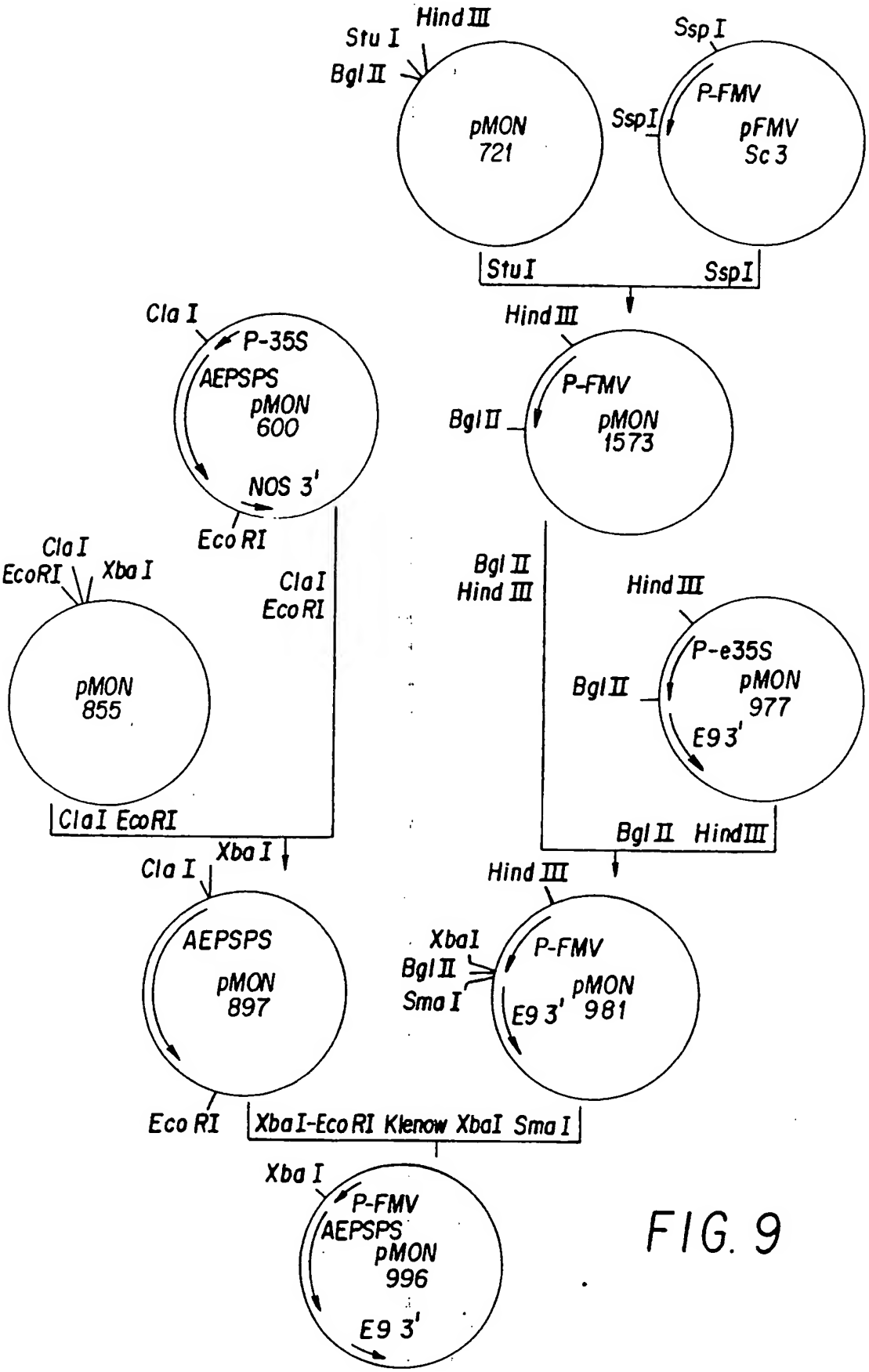
Hind III
Stu I
Bgl II
pMON 721
Ssp I
P-FMV
Ssp I
pFMV Sc 3
Stu I
Ssp I
Cla I
P-35S
AEPSPS
pMON 600
NOS 3'
Eco RI
Hind III
P-FMV
Bgl II
pMON 1573
Cla I
EcoRI
Xba I
pMON 855
Cla I
Eco RI
Cla I EcoRI
Bgl II
Hind III
Hind III
P-e35S
Bgl II
pMON 977
E9 3'
Bgl II Hind III
Xba I
Cla I
AEPSPS
pMON 897
Hind III
Xba I
Bgl II
Sma I
P-FMV
pMON 981
E9 3'
Eco RI
Xba I-Eco RI Klenow Xba I Sma I
Xba I
P-FMV
AEPSPS
pMON 996
E9 3'

FIG. 9

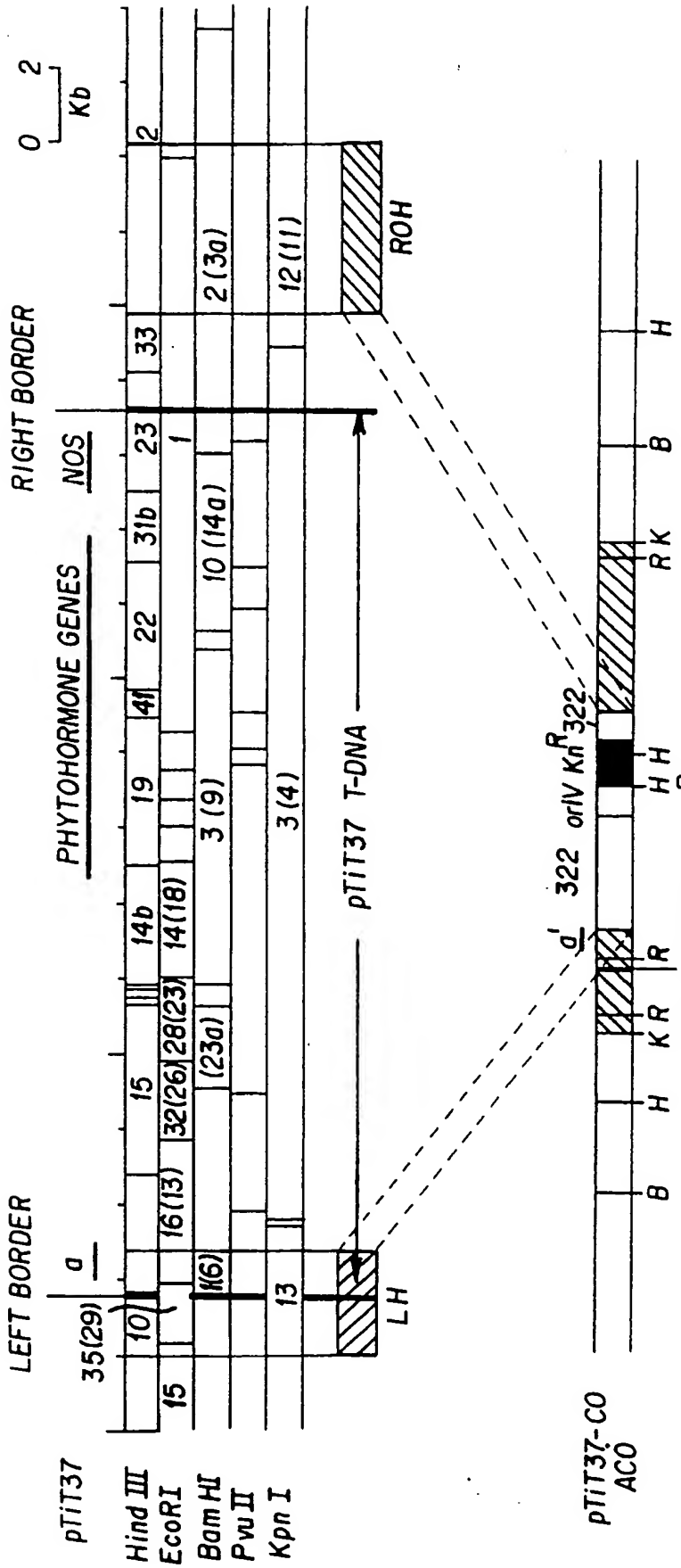
pTiT37
Hind III
EcoRI
Bam HI
Pvu II
Kpn I
LEFT BORDER
a
35(29)
10
15
16(13)
1(6)
13
15
32(26)
28(23)
(23a)
14b
14(18)
3 (9)
3 (4)
19
41
22
31b
10 (14a)
RIGHT BORDER
NOS
23
1
33
2 (3a)
12 (11)
2
0
2
Kb
PHYTOHORMONE GENES
ROH
pTiT37 T-DNA
LH
a'
322
oriV
Kn
R
322
pTiT37-CO
ACO
B
H
K
R
H
H
R
B
H
R
K
B
H
LEFT BORDER

FIG. 10

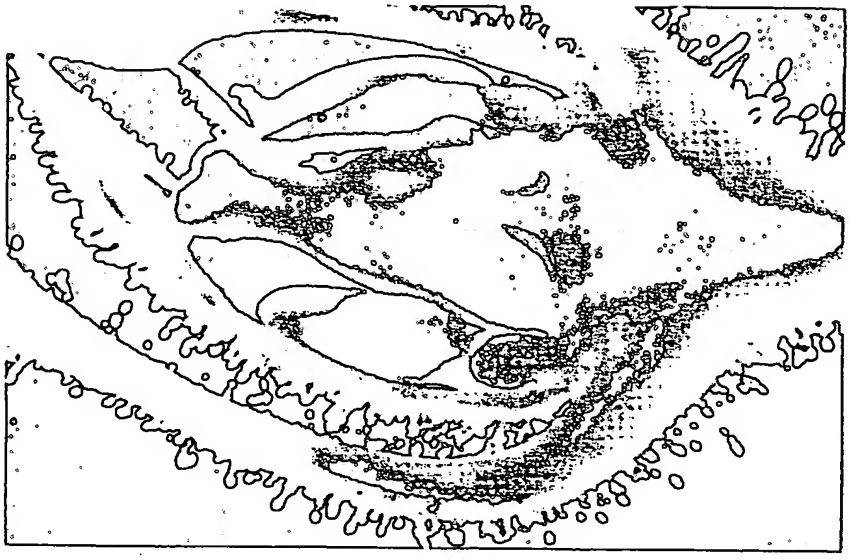

FIG.IIb

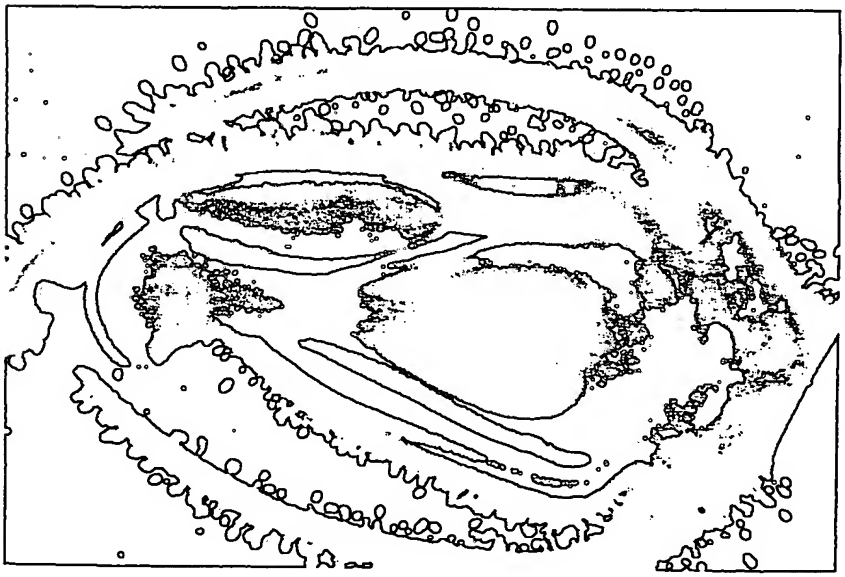

FIG.IIa

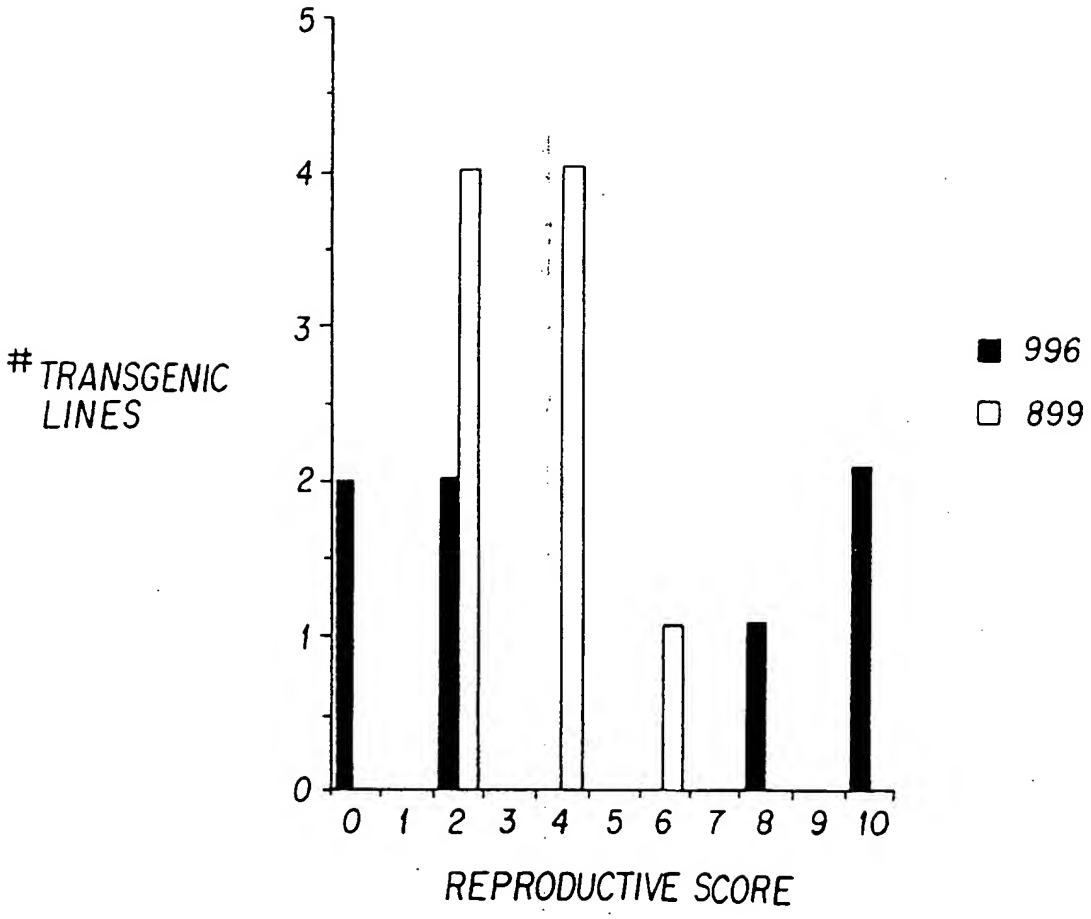
TRANSGENIC LINES
5
4
3
2
1
0
0 1 2 3 4 5 6 7 8 9 10
REPRODUCTIVE SCORE
996
899

FIG. 12